

Comparing the effects of different exogenous hormone combinations on seed-derived callus induction in *Nicotiana tabacum**

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Callus from *Nicotiana tabacum* is used as a model in plant developmental research. We tested several phytohormone (Indoleacetic acid – IAA; 2,4-Dichlorophenoxyacetic acid – 2,4-D; kinetin – KIN; 6-Benzylaminopurine – BAP) combinations to compare different approaches to callus induction directly from the seeds of *Nicotiana tabacum*. Callus formation was observed up to 4 weeks after sowing and the most effective were 0.5 mg/L of 2,4-D with 0.25 mg/L of BAP and 2 mg/L 2,4-D with 1 mg/L of BAP. The calli were green, photosynthetically active and after 6 weeks of growth, no stress symptoms (estimated on the basis of fluorescence of chlorophyll *a* in photosystem II) were noticed.

Key words: BAP, 2,4-D, auxin, cytokinin, callus, seed-derived callus, *Nicotiana tabacum*

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; BAP, 6-Benzylaminopurine; IAA, Indoleacetic acid; KIN, kinetin; PSII, photosystem II, F_{0v} , chlorophyll fluorescence in the absence of actinic (photosynthetic) light, in dark-adapted plants; $F_{m'}$, maximum of chlorophyll fluorescence in dark adapted plants $F_v/F_m = (F_m - F_0)/F_m$, maximum quantum yield of photosystem II. Phytohormone combinations: I, 2 mg/L IAA; II, 2 mg/L IAA with 1 mg/L KIN; IB, 2 mg/L IAA with 1 mg/L BAP; DB1, 0.5 mg/L 2,4-D with 0.25 mg/L BAP; DB2, 2 mg/L 2,4-D with 1 mg/L BAP; DB3, 1 mg/L 2,4-D with 0.5 mg/L BAP.

INTRODUCTION

Nicotiana tabacum is a model plant used in molecular biology research (Edwards *et al.*, 2017). Its callus serves as a model for studying cell differentiation (Bornman *et al.*, 1977) and embryogenesis (Afonso *et al.*, 1994; Srinivasan *et al.*, 2007; Pathi *et al.*, 2013; Li *et al.*, 2019). Phytohormones are chemicals that influence the growth and specialization of plant cells. Natural or synthetic phytohormones can be applied exogenously to alter plant development (Davies, 2010; Perianez-Rodriguez *et al.*, 2014; Jiang & Asami, 2019). Phytohormones (auxins and cytokinins, in particular) are utilized in plant biotechnology, e.g. for callus induction, preparation of a cell culture suspension or in micropropagation (Barkla *et al.*, 2014; Sello

et al., 2017; Efferth, 2019). Callus induction in *N. tabacum* and many other species is possible from various types of explants, such as the leaves and stems (Feldmann & Marks, 1986; Barkla *et al.*, 2014), fragments of seedlings (hypocotyls, cotyledons) (Nishiyama & Taira, 1966; Luo & Jia, 1998; Nieves & Aspuria, 2011; Chavan & Ahire, 2016) or germinating seeds and seedlings (Bennici *et al.*, 1972; Tabata & Hiraoka, 1976; Al-Khayri *et al.*, 1992; Gori *et al.*, 1998; Serrat *et al.*, 2014; Sello *et al.*, 2017). Using leaves and shoots as explants for the callus induction may be somewhat laborious, requiring healthy material to be selected and leaves to be carefully cut into pieces. If the *in vitro* material is not used, the sterilization process also poses a risk (Singh, 2019), as bleaching agents easily damage tissues, which increases mortality and decreases the success rate of callus induction. Using seeds may be easier, as in many cases the seed coat serves as a protection against both, the microorganisms and excessive sterilization agents. Thus, using germinating seeds for callus induction has many advantages, for example, it is much easier to sterilize seeds and callus induction is faster when compared to other types of explants (Al-Khayri *et al.*, 1992; Sello *et al.*, 2017; Singh, 2019). In our work, we examined several different combinations of various widely used phytohormones (IAA; 2,4-D; kinetin; BAP) to monitor changes in morphology and obtain an optimal protocol suitable for rapid mass induction of green calli from seeds and seedlings of *N. tabacum* cv. *Samsun*. According to our knowledge, such combinations have not been examined in previous studies. Callus formation was predominantly considered to be a result of the balance between cytokinins and auxins (Skoog and Miller, 1957; Gaspar *et al.*, 1996), and thus obtaining new protocols focused rather on finding the proper ratio (Ali *et al.*, 2007; Rahman *et al.*, 2010). Therefore, we assessed whether maintaining the ratio but changing the concentration of particular auxins and cytokinins affects the efficiency of callus induction in *N. tabacum*. The findings of this study indicate that the response to hormones is not only restricted to the ratio but also to the overall concentration of hormones. Our results may contribute to further research that focuses on the role of exogenous hormones on callus formation.

MATERIALS AND METHODS

Medium preparation and growth conditions. As plant material, the seeds of *Nicotiana tabacum* cv. *Samsun* were used. The seeds were surface sterilized using a solution of 30% (v/v) commercial bleach (ACE, Procter and Gamble) with an addition of 0.1% (v/v) Triton X-100

and vigorously shaken for 7 minutes. After surface sterilization, the seeds were rinsed 3–5 times using sterile distilled water. After sterilization, the seeds were inoculated on a solid (solidified with 1% w/v agar) MS medium (Murashige & Skoog, 1962; Duchefa Biochemie, Harlaam, Netherlands) supplemented with 3% (w/v) sucrose (Sello *et al.*, 2017). The plates with seeds were kept in a growth chamber at a temperature of 20°C, with white light illumination (fluorescent lamps, OSRAM L36W/77, Germany; photoperiod: 16L:8D; photon flux density – 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The seeds were sown on a control medium (MS medium supplemented with 3% (v/v) sucrose, without phytohormones) or a medium supplemented with a particular phytohormones – indoleacetic acid (IAA), kinetin (KIN), 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-Benzylaminopurine (BAP) combinations. Callus formation can be initiated in explants of *N. tabacum* using 2 mg/L IAA and 1 mg/L KIN as indicated in (Nishiyama & Taira, 1966). For *Arabidopsis thaliana*, a combination of 0.5 mg/L 2,4-D with 0.25 mg/L BAP was used as a successful callus inducing formula (Sello *et al.*, 2017). Therefore, we assumed these combinations would likewise be tested for *N. tabacum* (henceforth indicated as IK and DB1). On this basis, we tested some modifications of that protocol, using sole IAA (II) or changing KIN to BAP (thus, 2 mg/L IAA with 1 mg/L BAP – IB) or using 2,4-D instead of IAA (2 mg/L 2,4-D with 1 mg/L BAP – DB2). The latter resembles a well-known protocol for *N. tabacum* but utilizes hormones commonly used for *A. thaliana*. Finally, we examined the intermediate concentrations between the latter combination and a protocol from Sello *et al.* (2017), employing 1 mg/L of 2,4-D with 0.5 mg/L of BAP – DB3.

Fluorescence measurement. Dark-adapted (20 min) and 41-day old calli were examined for their photosystem II (PSII) efficiency (F_v/F_m) using OpenFluorCam FC 800-O (Photon Systems Instruments, Czech Republic) with a default protocol.

Statistical analysis. All experiments were conducted in three biological replicates and with at least 20 seeds per dish. The R software system (version 3.6.1) was used for statistical computing (R Core Team, 2019). After the Kruskal-Wallis test, multiple comparisons be-

tween combinations of different hormones (DB1, DB2, DB3, IB) in relation to the age of the plants were conducted using the `compare_means` function from the `ggpubr` package (version: 0.2.5, <https://CRAN.R-project.org/package=ggpubr>, A. Kassambara). To model count the data (number of seedlings and callus induced) we used generalized linear models with Poisson distributions (`glm` functions from `stats` packages). To compare the germination rates and F_v/F_m ratio after 41 days of growth, we set a control group in a callus formation medium DB1 (0.5 mg/L 2,4-D with 0.25 mg/L BAP) as the reference level. *P*-values below 0.05 indicated statistically significant differences. Full results of the regression modelling are available in the supplementary materials and at: https://osf.io/ktxns/?view_only=b3c27fe1d07542b5ba33e19aae8792e1

RESULTS AND DISCUSSION

Addition of hormones slightly decreased the germination rate assessed 4 weeks after sowing (Fig. 1A). The most explicit changes were observed for the DB1 and DB2 combinations. The differences were statistically significant when compared to control. Notably, these combinations of hormones seemed to have the most prominent effect on callus induction, as more than 40% of seedlings transformed into calli (Fig. 1B). Interestingly, the success rate of callus induction was below 20% in all IAA-treated samples. However, these differences were not statistically significant for medium II and IK when compared to other hormone combinations.

Exogenous phytohormones, regardless of their type and concentration, affected the growth of the seedlings and their morphology. We observed severe deformations, including significantly shortened hypocotyl and root, abnormally extended cotyledons and growth arrest (Fig. 2A–C and Fig. 2E–G). In particular, the latter was observed as a retardation of true leaf development in hormone-treated plants. Medium II had a different effect, as many of the seedlings developed true leaves. Medium II was sufficient to induce callus in *N. tabacum* which is consistent with other observations, e.g. in *Barleeria lupina* (Moin *et al.*, 2012). In the *Capiscum annuum* pep-

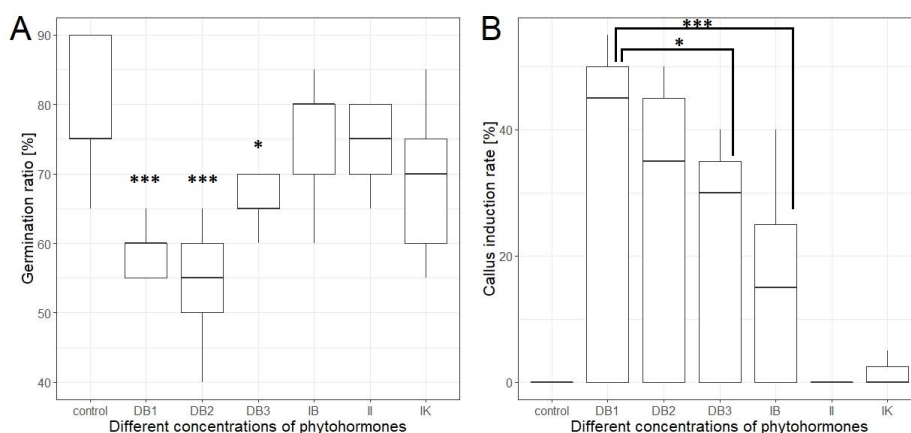


Figure 1. The effects of different concentrations of phytohormones on seed germination and calli induction.

Percentage of seeds (41st day) that germinated (A), of which some successfully transformed to calli (B), presented as a boxplot. Plants were grown on MS medium supplemented with 3% sucrose and hormones (DB1 – 0.5 mg/L 2,4-D with 0.25 mg/L BAP; DB2 – 2 mg/L 2,4-D with 1 mg/L BAP; DB3 – 1 mg/L 2,4-D with 0.5 mg/L BAP; IB – 2 mg/L IAA with 1 mg/L BAP; II – 2 mg/L IAA; IK – 2 mg/L IAA with 1 mg/L KIN); photoperiod: 16L:8D; photon flux density – 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Solid horizontal line represents the median, where the box is the interquartile range that covers the middle 50% of the data and whiskers – no more than 1.5 \times interquartile range (99% of all observations) from the edge of the box (extreme observations). Experiments were conducted in three biological replicates, with at least twenty plants. Statistically significant differences in comparison to control (A) or DB2, DB3 and IB (as no sufficient calli inductions were observed in the other groups and the control) to DB1 selected as the reference (B) are indicated with an asterisk ($p < 0.05$), a double asterisk ($p < 0.01$) or a triple asterisk ($p < 0.001$).

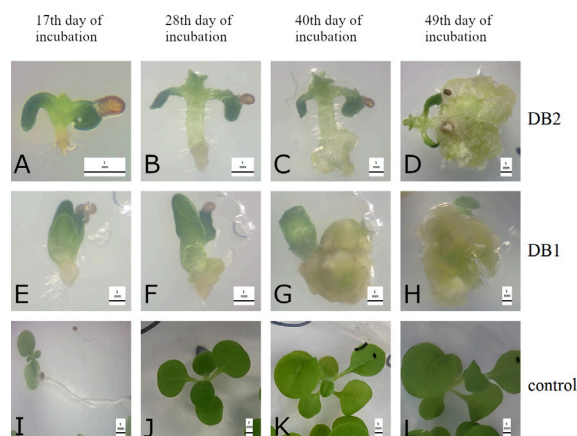


Figure 2. Morphology of seedlings transforming to calli in different callus-inducing media

Representative photos of seedlings growing on the DB2 (A–D) and DB1 (E–H) media with different combinations of hormones and the control (I–L). Plants were grown on MS medium supplemented with 3% sucrose and hormones; photoperiod: 16L:8D; photon flux density – $40 \mu\text{mol m}^{-2} \text{s}^{-1}$.

per pre-treatment with 2,4-D and subsequent treatment with IAA also induced calli (Gatz & Reinke, 2004). We conclude that medium II is not efficient enough for wide usage. Pale (white or yellow) leaf-like structures appeared in cultures treated with medium IB. However, this was a result of spontaneous morphogenesis of malformed hypocotyl and roots. These tissues formed colorless and friable calli during subsequent 4 weeks of development. Seedlings cultured on DB1, DB2 and DB3-culture media formed green calli after 40 days of growth (Fig. 2C, 2G). The size of the calli increased during further cultivation (Fig. 2D, 2H) and no spontaneous morphogenesis occurred.

Measurement of the fluorescence of chlorophyll *a* in PSII is the easiest and quickest way of assessing the capacity of photosynthesis and overall health of a plant. This method is widely used in the laboratory and field (Poudyal *et al.* 2018). We measured the minimal (F_0) and maximal (F_m) level of fluorescence for plants incubated in the dark. A high level of fluorescence is induced by a saturating pulse of light ($\sim 3500 \mu\text{mol m}^{-2} \text{s}^{-1}$). The ratio between the variable ($F_v = F_m - F_0$) and maximal fluorescence allows the maximal photochemical efficiency of PSII (F_v/F_m) to be estimated (Maxwell and Johnson, 2000). After four to six weeks of cultivation, all calli obtained (regardless of the hormone combination) were photosynthetically active and the quantum efficiency of PSII (F_v/F_m) was above 0.75 (Fig. 3). The F_v/F_m values for *N. tabacum* in soil cultures were above 0.8 (typically 0.82 ± 0.03) (Heggie *et al.*, 2005; Zhang *et al.*, 2011). The values of the F_v/F_m parameter are usually lower (down to 0.7) in *in vitro* cultures of *N. tabacum* and rise after transferring to *ex vitro* conditions (Pospíšilová *et al.* 1999). We observed a slight but statistically significant decrease in the F_v/F_m values for calli cultivated on IK, IB, DB2 or DB3 (p -vals < 0.028 ; Fig. 3). However, such a small change should not be considered to be a sign of significant deterioration in photosynthesis. No obvious symptoms of stress were observed. For medium II, we observed a statistically significant increase in the F_v/F_m value when compared to control.

DB3 was derived from DB1 and DB2 as an intermediate concentration, thus maintaining the hormone ratio.

Nevertheless, a change in the absolute concentration affected callus formation and it did not occur in a concentration-dependent manner. Our results indicate that the response to hormones is not only restricted to the ratio but also to the overall concentration of the hormones. Such observations may be explained as interaction with other factors and endogenous hormones. In garlic, the efficiency of callus formation depended on the interaction between exogenous and endogenous hormones, especially the jasmonic acid (Mostafa *et al.*, 2020). The number of calli of *Barringtonia racemosa* cultivated on MS medium increased in a straightforward manner solely with the 2,4-D concentration. A combination of both, KIN and 2,4-D had a different effect – the highest number of calli was observed for the lowest (1.0 mg/L) concentration of 2,4-D. Notably, maintaining the ratio (1:1) but using different KIN and 2,4-D concentrations (1; 1.5 or 2 mg/L) had, inversely, a concentration-dependent effect on the number of calli obtained (decrease from 38.3 to 8.3%). Different concentrations of KIN and 2,4-D administered at a constant ratio (1:1) induced formation of calli on the WPM medium, but the efficiency changed regardless of the concentration of hormones, with the lowest efficiency at 1.5 mg/L, which was an intermediate concentration (Osman *et al.*, 2016). We emphasize that changing the concentrations of auxins and cytokinins, while maintaining their constant ratio, plays an important role in the callus induction and should be considered in planning new experiments or optimizing new protocols. Our observations raise the question as to exactly how the exogenous hormones initiate a transformation of tissue to the callus and how the cross-talk with numerous signaling pathways works.

The results of our study show that it is possible to induce callus from the seedlings of *N. tabacum* var. *Samsun* by sowing the surface-sterilized seeds on a hormone-amended medium. This method proved to be

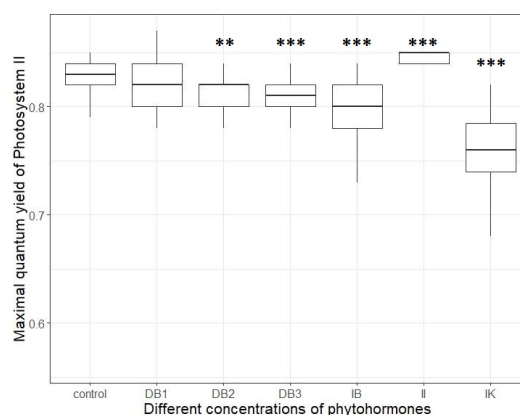


Figure 3. The maximum quantum efficiency of PSII of the calli induced

Values of F_v/F_m (41st day) presented as a boxplot. Plants were grown on MS medium supplemented with 3% sucrose and hormones (DB1 – 0.5 mg/L 2,4-D with 0.25 mg/L BAP; DB2 – 2 mg/L 2,4-D with 1 mg/L BAP; DB3 – 1 mg/L 2,4-D with 0.5 mg/L BAP; IB – 2 mg/L IAA with 1 mg/L BAP; II – 2 mg/L IAA with 1 mg/L KIN); photoperiod: 16L:8D; photon flux density – $40 \mu\text{mol m}^{-2} \text{s}^{-1}$. Solid horizontal line represents the median, where the box is the interquartile range that covers the middle 50% of the data and whiskers – no more than $1.5 \times$ interquartile range (99% of all observations) from the edge of the box (extreme observations). Experiments were conducted in three biological replicates, with at least twenty plants. Statistically significant differences in comparison to the control are indicated with a double asterisk ($p < 0.01$) or triple asterisk ($p < 0.001$).

quick and efficient, as the calli were ready for further use as intended only 4.5 weeks after sowing. The main advantage of this approach is its relative simplicity and the low mortality of explants. Additionally, it is easy to tell if transformation starts, as the seedling growth is stopped, and hypocotyl and the root are characteristically shortened. Our method is suitable for mass production of calli and may increase the popularity of the use of germinating seeds as a source of calli. We believe that a combination of 0.5 mg/L 2,4-D with 0.25 mg/L BAP or 2 mg/L 2,4-D with 1 mg/L BAP (DB1 and DB2, respectively) was optimal and we recommend its use. Importantly, we provide information on how many seeds are sufficient to induce the desired number of calli. When planning an experiment, we recommend sowing twice as many seeds as the desired number of calli.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding publication of this article.

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